

## $\alpha$ -AMINOPHOSPHONOUS ACIDS: THE SUBSTRATES OF ATP-PP<sub>i</sub> EXCHANGE REACTION, CATALYSED BY AMINOACYL-tRNA SYNTHETASES

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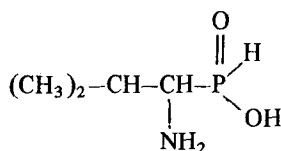
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### 1. Introduction

Studies on the specificity of aminoacyl-tRNA synthetases towards amino acid substrates have led to the conclusion that the  $\alpha$ -carboxyl group of the substrate plays a secondary role in the binding with the enzymes [1]. Usually these studies involved such amino acid derivatives (e.g., esters, amides, hydrazides) [2] which could not participate in the activation reaction. However, substitution of the COOH group in the substrate amino acid by a phosphonyl group  $P(O)(OH)_2$  resulted in a sharp decrease (by 2–3 orders of magnitude) of the affinity for the enzyme [3], and these aminophosphonic acids were not activated by synthetases [4]. Therefore, the specificity of synthetases towards the COOH groups of substrates requires further investigations.

This work demonstrates that  $\alpha$ -aminophosphonous acids (I) and (II) possess a high affinity for valyl- and methionyl-tRNA-synthetases, respectively, and are substrates in the ATP-PP<sub>i</sub> exchange reaction.



I

$\alpha$ -aminoisobutylphosphonous acid

### 2. Materials and methods

#### 2.1. Chemical compounds

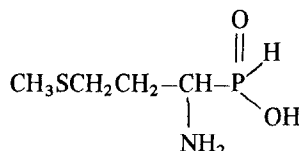
Standard <sup>14</sup>C-labelled amino acids were obtained from Chemapol (Czechoslovakia) and Amersham (England), and sodium [<sup>32</sup>P]pyrophosphate, from Isotope (USSR). Phosphonous acids (I) and (II) were synthesized according to [5].

#### 2.2. Valyl- and methionyl-tRNA-synthetases

These were isolated from *E. coli* B according to [6] and [7], respectively. Total *E. coli* tRNA was prepared as in [8]. *E. coli* B tRNA<sup>Val</sup> and tRNA<sup>Met</sup> were purified by the method in [9] with subsequent separation on a Sepharose 4B column [10].

#### 2.3. Determination of synthetase and acceptor activity

(a) The activity of synthetases was determined using the ATP-PP<sub>i</sub> exchange reaction according to [11]. Samples were analysed as in [7].



II

$\alpha$ -amino- $\gamma$ -methylthiopropylphosphonous acid

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(b) The acceptor activity of tRNA<sup>Val</sup> and tRNA<sup>Met</sup> was assayed as in [11]. Incubation samples (0.5 ml) contained ( $\mu\text{mol}$ ): Tris/HCl, pH 8.0, 50;  $\text{Mg}(\text{CH}_3\text{COO})_2$ , 5; KCl, 5; ATP, 1;  $\beta$ -mercaptoethanol, 2;  $^{14}\text{C}$ -labelled amino acids, 0.001; tRNA, 0.01; and 0.005–0.01 mg corresponding enzyme. The mixture was incubated for 10 min at 37°C.

#### 2.4. The effect of I and II on the activity of synthetases

Aqueous solutions of I and II were freshly prepared prior to use and the aliquots were added to the standard samples for ATP–PP<sub>i</sub> exchange or tRNA aminoacylation, immediately before the addition of the enzyme. The enzyme activity was assayed according to (a) and (b) of 2.3, respectively.

#### 2.5. Determination of kinetic constants

The  $K_m$  and  $V_{\max}$  values for each amino acid substrate and  $\alpha$ -aminophosphonous acids as well as the  $K_i$  value for each inhibitor, were determined from Lineweaver-Burk reciprocal plots of the data obtained in the standard ATP– $^{32}\text{P}$ PP<sub>i</sub> exchange and tRNA aminoacylation assays. No less than six points were used for each line in the plot and all the plots were done at least in duplicate. The values of  $V_{\max}$  were determined in the presence of fixed concentrations of the enzyme,  $\text{Mg}^{2+}$  ions and ATP at constant pH. The apparent  $K_m$  and  $K_i$  values were compared with those of  $K_m$  for amino acids which were used to characterize the affinity of the substrate for the enzyme.

#### 2.6. Determination of radioactivity

The radioactivity of samples was measured with an SL-30 liquid-scintillation counter combined with a Multi-20 computer in the Multimat system (Inter-technique, France).

### 3. Results and discussion

Compounds I and II are the first representatives of a new class of amino acid analogues,  $\alpha$ -aminophosphonous acids, discovered in this laboratory. They are similar to aminocarboxylic acids in a number of properties. Therefore, enzymatic experiments followed the standard scheme [4].

Table 1  
Comparison of kinetic parameters of valine and methionine with those of their phosphonous analogues

| Compounds                             | $K_m$ (M)            | $K_i$ (M)          | $V_{\max}$ |
|---------------------------------------|----------------------|--------------------|------------|
| tRNA aminoacylation reaction          |                      |                    |            |
| L-Valine                              | $3 \times 10^{-5}$   |                    |            |
| I                                     |                      | $2 \times 10^{-4}$ |            |
| L-Methionine                          | $3.4 \times 10^{-5}$ |                    |            |
| II                                    |                      | $6 \times 10^{-4}$ |            |
| ATP–PP <sub>i</sub> exchange reaction |                      |                    |            |
| L-valine                              | $2.1 \times 10^{-4}$ |                    | 1          |
| I                                     | $8.3 \times 10^{-4}$ |                    | 0.12       |
| L-methionine                          | $6.0 \times 10^{-5}$ |                    | 1          |
| II                                    | $1.0 \times 10^{-3}$ |                    | 0.54       |

Compounds I and II were effective and specific inhibitors of the reaction of tRNA aminoacylation catalysed by valyl- and methionyl-tRNA-synthetases (table 1). The inhibition was reversible and competitive towards the substrates, L-valine and L-methionine (fig.1).

From the chemical point of view it seemed quite

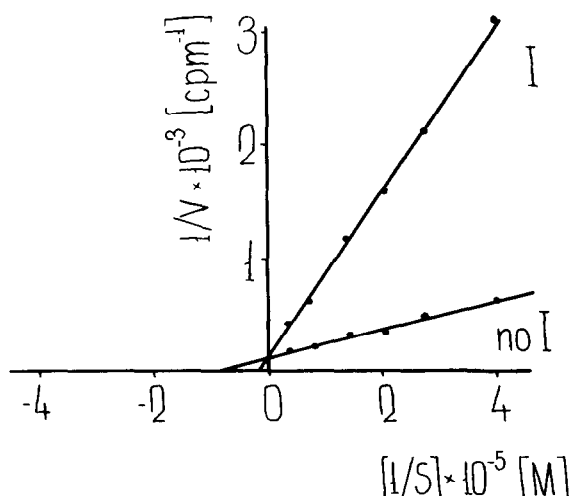


Fig.1. Lineweaver-Burk plot for the inhibition of aminoacylation of tRNA<sup>Val</sup> by I ( $5.5 \times 10^{-4}$  M) measured by varying the  $^{14}\text{C}$  valine concentration in the presence of saturating amounts of ATP ( $2 \times 10^{-3}$  M). The conditions for inhibition are described in section 2.

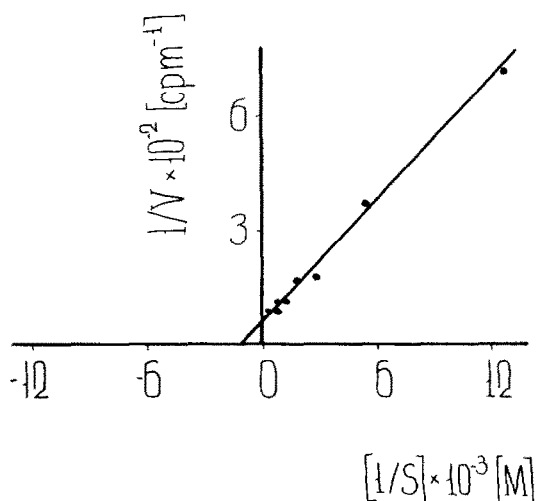
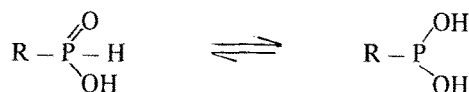


Fig.2. Determination of the Michaelis constant  $K_m$  for I in the reaction of ATP-PP<sub>i</sub> exchange. Samples contained in 0.5 ml: Tris/HCl buffer, pH 8.0 ( $5 \times 10^{-2}$  M), 0.01 mg enzyme, Mg<sup>2+</sup> ( $2.3 \times 10^{-3}$  M), NH<sub>4</sub><sup>+</sup> ( $3.5 \times 10^{-2}$  M), ATP ( $9 \times 10^{-4}$  M), <sup>32</sup>PP<sub>i</sub> ( $2 \times 10^{-3}$  M), β-mercaptoethanol ( $2.5 \times 10^{-3}$  M) and I in the concentration range of  $3 \times 10^{-5}$  to  $6 \times 10^{-5}$  M. Incubation for 30 min at 37°C.

plausible that phosphonous acids would react with ATP yielding corresponding adenylates. Compounds I and II were found to be capable of substituting for L-valine and L-methionine in the ATP-PP<sub>i</sub> exchange reactions catalysed by the corresponding synthetases, their affinities for the enzymes and the  $V_{max}$  of the reactions being only several times lower than those of the substrates (table 1, fig.2). It is relevant to point out that I and II are racemates and their effectiveness therefore is in fact still higher.

The possibility that the ATP-PP<sub>i</sub> exchange reaction for I and II might depend either on the nucleophilicity of the  $\text{--P(=O)(OH)H}$  group or on its steric parameters. Aminophosphonic acids cannot be involved in the reaction of ATP-PP<sub>i</sub> exchange [4], however, their anhydrides with AMP, aminophosphonyl adenylates, prepared recently are effective inhibitors of synthetases [13]. Therefore, the similarity between the steric parameters of the  $\text{--P(=O)(OH)H}$  and  $\text{--C(=O)OH}$  groups seems to

be preferable. Phosphonous acids can exist in tautomeric forms:



However, under normal conditions, the equilibrium is almost completely shifted to the four-coordinated structure and the forms shown for I and II seem to be those participating in the enzymatic reaction.

The enzymatic activation of I and II suggests that these could be involved in the complete reaction. This possibility and the role of enzymatic deacylation in the process are being studied in our laboratory using new inhibitors of deacylation [14].

Therefore, compounds I and II are the first illustration of the participation of functional groups other than the carboxyl one in the activation reaction.

## References

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